DECLARATION

I, Emiko Oku of HIROTA & ASSOCIATES, residing at Wakabayashi Bldg. 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed for a patent application in Japan under No. 11-240642 on August 27, 1999 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION in Tokyo, Japan, entitled: "HIGH-AFFINITY CHOLINE TRANSPORTER".

Signed this 29 day of August, 2006

Emiko Oku

Emiko Oku

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【Confirmation of Proof】　　Proof Necessary

【Name of Document】 SPECIFICATION

【Title of the Invention】 HIGH-AFFINITY CHOLINE TRANSPORTER

【Scope of Claims】

【Claim 1】 A gene which encodes a protein (a) or (b) described below;

(a) a protein comprising an amino acid sequence represented by Seq. ID No. 2,

(b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having high-affinity choline transporter activity.

【Claim 2】 DNA containing a base sequence represented by Seq. ID No. 1 or its complementary sequence and a part or a whole of these sequences.

【Claim 3】 DNA derived from a nematode which hybridizes with DNA according to claim 2 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

【Claim 4】 A gene which encodes a protein (a) or (b) described below;

(a) a protein comprising an amino acid sequence represented by Seq. ID No. 4,

(b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having high-affinity choline transporter activity.

【Claim 5】 DNA containing a base sequence represented by Seq. ID No. 3 or its complementary sequence and a part or a whole of these sequences.

【Claim 6】 DNA derived from a rat which hybridizes with DNA comprising a gene according to claim 5 under a stringent condition,

and encodes a protein having high-affinity choline transporter activity.

【Claim 7】 A gene which encodes a protein (a) or (b) described below;

(a) a protein comprising an amino acid sequence represented by Seq. ID No. 6,

(b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having high-affinity choline transporter activity.

【Claim 8】 DNA containing a base sequence represented by Seq. ID No. 5 or its complementary sequence and a part or a whole of these sequences.

【Claim 9】 DNA derived from a human which hybridizes with DNA comprising a gene according to claim 8 under a stringent condition, and encodes a protein having high-affinity choline transporter activity.

【Claim 10】 A protein comprising an amino acid sequence represented by Seq. ID No. 2 and having nematode high-affinity choline transporter activity.

【Claim 11】 A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having nematode high-affinity choline transporter activity.

【Claim 12】 A protein comprising an amino acid sequence represented by Seq. ID No. 4 and having rat high-affinity choline transporter activity.

【Claim 13】 A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having

rat high-affinity choline transporter activity.

[Claim 14] A protein comprising an amino acid sequence represented by Seq. ID No. 6 and having human high-affinity choline transporter activity.

[Claim 15] A protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No. 6, and having human high-affinity choline transporter activity.

[Claim 16] An antibody that specifically binds to the protein having nematode high-affinity choline transporter activity according to claim 10 or 11.

[Claim 17] An antibody that specifically binds to the protein having rat high-affinity choline transporter activity according to claim 12 or 13.

[Claim 18] An antibody that specifically binds to the protein having human high-affinity choline transporter activity according to claim 14 or 15.

[Claim 19] A screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in evaluating high-affinity choline transporter activity of the protein having high-affinity choline transporter activity according to any one of claims 10 to 15 in the presence of a subject material.

[Claim 20] A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell which can express the protein having high-affinity choline transporter activity according to any one of claims 10 to 15 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount

of a protein having high-affinity choline transporter activity in the cell is evaluated.

[Claim 21] A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity according to any one of claims 10 to 15 in a cell which can express a protein having high-affinity choline transporter activity.

[Claim 22] A preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene or the DNA according to any one of claims 7 to 9 into a cell whose function of a high-affinity choline transporter gene is deficient on its chromosome.

[Claim 23] The preparing method of a cell having high-affinity choline transporter activity according to claim 22, wherein the cell having high-affinity choline transporter activity is integrated with the gene or the DNA according to any one of claims 7 to 9 in its chromosome, and stably shows high-affinity choline transporter activity.

[Claim 24] A cell having high-affinity choline transporter activity being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 22 or 23.

[Claim 25] A non-human animal whose function of a high-affinity choline transporter gene is deficient or overexpresses on its chromosome.

[Claim 26] The non-human animal according to claim 25, wherein the non-human animal is a mouse or a rat.

[Claim 27] A screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to the non-human animal according to claim 25 or 26.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

This invention relates to a protein having high-affinity choline transporter activity, a gene encoding said protein and the use of the same.

[0002]

[Prior Art]

The autonomic nervous system which spreads to organs throughout a body and regulates the most basic functions of living organism including energy metabolism, circulation, respiration and reproduction along with endocrine system, is classified into the sympathetic and parasympathetic nervous systems. All autonomic nerve fibers excluding postganglionic fibers of the sympathetic nerve, motor nerve fiber, and sudoriferous gland/blood vessel dilative fiber in the sympathetic nerve are cholinergic, and acetylcholine is vital for the function of the autonomic nerve and the motor nerve. It has been known that the cholinergic neuron, being observed also in the brain, is important for recognizing function of the brain and that it degenerates after the onset of Alzheimer's disease. In the cholinergic neuron, because of lack of biosynthetic ability for choline, choline, an acetylcholine decomposition product, is taken up into a cell by a high-affinity choline transporter at the presynaptic terminals to be reused for synthesizing

acetylcholine. The high-affinity choline uptake is a rate-limiting step for acetylcholine synthesis and is presumed to regulate the efficiency of synaptic transmission (J. Neurochem. 18, 781-798, 1971, Science 178, 626-628, 1972, Biochem. Biophys. Acta 291, 564-575, 1973, Mol. Pharmacol. 9, 630-639, 1973, J. Pharmacol. Exp. Ther. 192, 86-94, 1975, J. Neurochem. 30, 15-21, 1978, J. Neurochem. 44, 11-24, 1985, J. Neurochem. 60, 1191-1201, 1993, J. Neurochem. 20, 581-593, 1973, Eur. J. Pharmacol. 102, 369-370, 1984). To date, most of cDNAs of transporters for major neurotransmitters have been isolated, however, a cDNA of the high-affinity choline transporter, which is physiologically important, has not been identified.

【0003】

【An Object to be Attained】

So far, the existence of a protein being localized in the cholinergic neuron and having a function of taking up choline, a precursor of acetylcholine, into a cell has been expected, but molecular properties of said protein, a high-affinity choline transporter, have been unknown. An object of the present invention is to provide a physiologically important protein having the high-affinity choline transporter activity, a gene which encodes the protein, and a screening method of a high-affinity choline transporter activity promoter using the protein, the gene and the like.

【0004】

【Means to Attain the Object】

The inventors have conducted intensive study to attain the above-mentioned object: with information of genomic project (Science 282, 2012-2018, 1998), Na^+-dependent transporter cDNAs being expected from the genomic sequence of a nematode (C.

elegans) were cloned one by one, and the high-affinity choline uptake activity of each cDNA was examined in the oocyte expression system of Xenopus, and the cDNA of nematode high-affinity choline transporter (cho-1) was identified on the basis of the above examination, then homologous molecules (CHT1) were cloned from rat spinal cord by using the homology of a base sequence to the cDNA as an index. This CHT1 had no homology to neurotransmitter transporters (J. Neurochem. 71, 1785-1803, 1998), but had 20 to 25% homology to molecules which belong to Na^+-dependent glucose transporter family (Nature 330, 379-381, 1987).

[0005]

Northern blot analysis revealed that transcripts of CHT1 were confirmed only in spinal cord, basal forebrain, corpus striatum and brain stem, and CHT1 seemed to be expressed in cholinergic neurons. Accordingly, CHT1 was expressed in oocytes of Xenopus. As a result, choline uptake activity that is Na^+-dependent and completely inhibited by hemicholinium-3 was observed. These results indicate that CHT1 has high-affinity choline transporter activity. Further, the inventors have cloned choline transporter cDNAs derived from a human, and determined their base sequences, and have confirmed that their expression products have high-affinity choline uptake activity. The present invention has thus completed.

[0006]

The present invention relates to a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 2, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having high-affinity choline

transporter activity (claim 1), DNA containing a base sequence represented by Seq. ID No. 1 or its complementary sequence and a part or a whole of these sequences (claim 2), DNA derived from a nematode which hybridizes with DNA according to claim 2 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 3), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 4, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having high-affinity choline transporter activity. (claim 4), DNA containing a base sequence represented by Seq. ID No. 3 or its complementary sequence and a part or a whole of these sequences (claim 5), DNA derived from a rat which hybridizes with DNA comprising a gene according to claim 5 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 6), a gene which encodes a protein (a) or (b) described below; (a) a protein comprising an amino acid sequence represented by Seq. ID No. 6, (b) a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having high-affinity choline transporter activity (claim 7), DNA containing a base sequence represented by Seq. ID No. 5 or its complementary sequence and a part or a whole of these sequences (claim 8), DNA derived from a human which hybridizes with DNA comprising a gene according to claim 8 under a stringent condition, and encodes a protein having high-affinity choline transporter activity (claim 9), a protein comprising an amino acid sequence represented by Seq. ID No. 2 and having nematode high-affinity

choline transporter activity (claim 10), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.2, and having nematode high-affinity choline transporter activity(claim 11), a protein comprising an amino acid sequence represented by Seq. ID No. 4 and having rat high-affinity choline transporter activity (claim 12), a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.4, and having rat high-affinity choline transporter activity (claim 13), a protein comprising an amino acid sequence represented by Seq. ID No. 6 and having human high-affinity choline transporter activity (claim 14), and a protein comprising an amino acid sequence where one or a few amino acids are deficient, substituted or added in the amino acid sequence represented by Seq. ID No.6, and having human high-affinity choline transporter activity (claim 15).

[0007]

The present invention also relates to an antibody that specifically binds to the protein having nematode high-affinity choline transporter activity according to claim 10 or 11 (claim 16), an An antibody that specifically binds to the protein having rat high-affinity choline transporter activity according to claim 12 or 13 (claim 17), and an antibody that specifically binds to the protein having human high-affinity choline transporter activity according to claim 14 or 15 (claim 18).

[0008]

The present invention further relates to a screening method of a promoter or a suppressor of high-affinity choline transporter activity characterized in evaluating high-affinity

choline transporter activity of the protein having high-affinity choline transporter activity according to any one of claims 10 to 15 in the presence of a subject material (claim 19), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in comprising the steps of: a cell which can express the protein having high-affinity choline transporter activity according to any one of claims 10 to 15 is cultivated in vitro in the presence of a subject material; the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is evaluated (claim 20), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to a non-human animal and then evaluating the activity and/or the expression amount of a protein having high-affinity choline transporter activity according to any one of claims 10 to 15 in a cell which can express a protein having high-affinity choline transporter activity (claim 21), a preparing method of a cell having high-affinity choline transporter activity characterized in introducing the gene or the DNA according to any one of claims 7 to 9 into a cell whose function of a high-affinity choline transporter gene is deficient on its chromosome (claim 22), the preparing method of a cell having high-affinity choline transporter activity according to claim 22, wherein the cell having high-affinity choline transporter activity is integrated with the gene or the DNA according to any one of claims 7 to 9 in its chromosome, and stably shows high-affinity choline transporter activity (claim 23), a cell having high-affinity choline transporter activity

being obtainable by the preparing method of a cell having high-affinity choline transporter activity according to claim 22 or 23 (claim 24), a non-human animal whose function of a high-affinity choline transporter gene is deficient or overexpresses on its chromosome (claim 25), the non-human animal according to claim 25, wherein the non-human animal is a mouse or a rat (claim 26), a screening method of a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression characterized in administering a subject material to the non-human animal according to claim 25 or 26 (claim 27).

[0009]

[Mode for Carrying out the Invention]

The cDNA of nematode high-affinity choline transporter of the present invention, being described in Seq. ID No. 1, can be obtained by injecting each cRNA prepared from candidate full-length cDNAs, which are expected as a member of Na^+-dependent transporter family according to C. elegans genome project, into oocytes of Xenopus, and examining the uptake of choline. The high-affinity uptake of choline in brain synaptosomes of mammals was completely inhibited by 1 μMhemicholinium-3 (HC3) (Ki=10-100 nM), while the low-affinity uptake of choline, which is distributed in every cells, was inhibited only by HC3 with higher concentration (Ki=50 μM). Therefore, the sensitivity to 1 μM HC3 can be used as criteria of high-affinity choline uptake during the process. For example, it is possible to confirm the identification, the expression, and the localization of an object gene from the candidate cDNA of C. elegans as follows.

[0010]

It has been found that cDNA corresponding to the gene

expected as C48D1.3 promotes significant choline uptake, being inhibited by 1 μM HC3, in the high-affinity choline uptake process. Fig. 1 shows the result of [^{3}H] choline uptake of oocytes from Xenopus being injected with C48D1.3 cRNA or water. In Fig. 1, the closed and the open columns indicate choline uptake in the absence or the presence of 1 μM HC3 respectively, and each column is shown by mean ± SEM (n=6 to 8 oocytes). Fig.2 shows the effect of Na^+ on the choline uptake, and the closed columns indicate choline uptake measured in the standard solution ([Na^+]=100 mM), the open columns indicate choline uptake in the absence of Na^+ (Na^+ was substituted with Li^+). In addition, Fig. 3 shows the inhibition of choline uptake induced by HC3. Based on the above-mentioned Fig. 2 and 3, it is presumed that the uptake is Na^+-dependent, and that Ki of HC3 is 50 nM. The cDNA clone was designated as cho-1 (high-affinity choline transporter-1).

[0011]

By comparing a base sequence of cDNA and that of genome, cho-1 gene was found to comprise 9 exons. A protein expected from a base sequence of cDNA of cho-1 includes 576 amino acid residues (see Fig. 4), and this protein, being represented by Seq. ID No. 2, can be constructed by a usual method. When the available data base was searched, the amino acid sequences of cho-1 showed weak, but significant homology to members of Na^+-dependent glucose transporter family. Hydrophobic analysis and comparison to other transporters suggest that there is a twelve-transmembrane region (see Fig. 7).

[0012]

Then, in order to identify cells expressing cho-1 in the nervous system of C. elegans, a gene of a green fluorescent protein (GFP) fused with a region 5.1kb upstream from cho-1 gene was

introduced into a nematode, and distribution of neurons expressing cho-1::gfp was examined. A photograph of L1 larva possessing cho-1::gfp reporter DNA at the outside of chromosome is shown as Fig. 5 (scale bar; 50 μm). In Fig. 5, the arrowhead indicates nerve ring. In the ventral nerve cord, GFP is expressed only in cholinergic motor nerve, however, some of DA, DB nerve cells do not express GFP owing probably to deficiency of reporter DNA at the outside of chromosome. It supports the idea that cho-1 is a high-affinity choline transporter of the cholinergic neuron.

[0013]

The cDNA of rat high-affinity choline transporter of the present invention, being described in Seq. ID No. 3, can be prepared, for example, by a method comprising the steps of: paying attention to cho-1 homologous molecules of vertebrates and searching data base with amino acid sequences expected from cho-1, and identifying one candidate (GenBank accession number AQ 316435) in human genomic survey sequence (GSS); amplifying cDNA fragments from rat spinal cord cDNA by PCR with degenerate primers on the basis of homology of base sequences between the human genome DNA and cho-1; screening rat spinal cord cDNA library with this fragment, and a positive cDNA clone was obtained. A protein with 580 amino acid residues showing 51% identity and 70% similarity to cho-1 was expected from the base sequence of the longest reading frame (see Fig. 4). This rat cDNA clone was designated as CHT1. In Fig. 4, each amino acid sequence of rat CHT1 and C. elegans CHO-1 is shown, and the identical and the similar residues are indicated on a black ground and a gray ground respectively. The expected transmembrane region I-XII is underlined. This protein represented by Seq. ID No.

4 can be constructed by a usual method.

[0014]

The above-mentioned amino acid sequence of CHT1 is significantly homologous to members of Na^+-dependent glucose transporter family (20 to 25%). The phylogenetic tree of Na^+-dependent glucose transporter family made by neighbor-joining method using a program CLUSTALW of National Institute of Genetics (Mishima, Japan) is shown in Fig. 6. In Fig. 6, the percentage of the identical amino acids, being contained in each protein, to rat CHT1 is shown on the right side. On the other hand, no homology was observed to a yeast choline transporter (J. Biol. Chem. 265, 15996-16003, 1990), a creatine transporter which had been originally reported as a high-affinity choline transporter (Biochem. Biophys. Res. Commun. 198, 637-645, 1994), and other neurotransmitter transporters.

[0015]

The expected topology of CHT1 is thought to be the same as that of C. elegans CHO-1 fundamentally. Fig. 7 shows the expected topology of rat CHT1. In Fig. 7, the closed circles indicate the identical residues, the shadowed circles indicate highly conserved residues, and open circles indicate nonsimilar residues. The offshoots indicate the expected glycosylation sites. P among the circles shows the expected parts of phosphorylation induced by protein kinase C.

[0016]

Next, the distribution of CHT1 mRNA expression was examined by Northern blot analysis and *in situ* hybridization. The expression of transcripts with the length of about 5 kb was confirmed by Northern blot analysis of various tissues of rats.

Fig. 8 shows the result of Northern blot analysis of mRNA transcript of CHT1 in rat tissue, and the length of RNA standard (0.24 to 9.5 kb; GIBCO BRL) is exhibited on the left side. As shown in Fig. 8, an abundance of transcripts were confirmed in basal forebrain, brain stem and spinal cord, and a little of those were confirmed in corpus striatum. These tissues are known to contain cholinergic neurons. On the other hand, no transcript was observed in other regions of the brain or in tissues of non-nervous systems.

[0017]

Consistent with these results, *in situ* hybridization confirmed the expression of CHT1 mRNA in cell groups of main cholinergic neurons including corpus striatum, cell population in basal forebrain and ventral horn in spinal cord. Fig. 9 and 10 (scale bar; 1 mm) show micrographs of sections in bright-field, which were hybridized with a cRNA probe of an antisense labeled by digoxigenin. These micrographs relate to in situ hybridization analysis of CHT1 transcripts in rat brain and spinal cord. Fig. 9 indicates that mRNA transcripts of CHT1 were detected in vertical and horizontal limbs of the diagonal band (VDB, HDB), medial septal nucleus (MS), caudate and putamen (Cpu), and olfactory tubercle (Tu). Fig. 10 indicates that the expression was observed in ventral horn (VH) in spinal cord. Further, the adjacent section hybridized with a probe of vesicle acetylcholine transporter showed essentially same distribution. This expression distribution is essentially same as the reported distribution of cholineacetyl group transferase or vesicle acetylcholine transporter. These results show that the expression of CHT1 mRNA is limited to cholinergic neurons.

[0018]

Next, choline uptake of CHT1 was examined by using oocytes of Xenopus. The choline uptake of the oocytes injected with CHT1 cRNA was 2 times to 4 times more than that of controls injected with water. Fig. 11 shows the result of [^{3}H] choline uptake of oocytes of Xenopus injected with CHT1 cRNA or water. In Fig. 11, the open and the closed columns respectively indicate choline uptake in the standard solutions containing 100 mM NaCl or LiCl, and each column is shown by mean ± SEM (n=6 to 8 oocytes). The effect of choline concentration on choline uptake is shown in Fig. 12. In Fig. 12, choline uptake of oocytes injected with water was subtracted from that of oocytes injected with cRNA in order to figure out CHT1-induced choline uptake, and the choline uptake was fitted to Michaelis-Menten curve. As shown in Fig. 12, choline uptake of CHT1 saturated when increasing choline concentration (Km=2.2 ± 0.2 μM, n=3).

【0019】

Then, the result of HC3-induced inhibition of choline uptake is shown in Fig. 13. Fig. 13 indicates that the Km of endogenous choline uptake of control is higher than 10 μM and that choline uptake of CHT1 is completely inhibited by 0.1 μM HC3 (Ki=2-3 nM), whereas 10 μM HC3 induced only slight inhibition in control. As shown in Fig. 14, ion-dependency of choline uptake of CHT1 was examined and found to be Cl^--dependent as well as Na^+-dependent. The closed and the open columns indicate choline uptake of oocytes injected with water and with cRNA respectively (100 mM NaCl in the standard solution is substituted with 100 mM of each salt) shown in the figure. These results indicate that CHT1 has the characteristics expected from high-affinity choline uptake in brain synaptosomes (high-affinity to choline, high sensitivity to HC3, and Na^+-Cl^--dependency) (J. Neurochem.

27, 93-99, 1976).

[0020]

In addition, [^{3}H] HC3 binding activity of membranes prepared from COS7 cells introduced with CHT1 cDNA and a vector (control) respectively was examined. The result is shown in Fig. 15. As Fig. 15 indicates, Na^+-dependent [^{3}H] HC3 binding was observed in a membrane of a cell where CHT1 was expressed, but not in a control membrane. Subsequently, a saturation analysis was conducted for specific [^{3}H] HC3 binding. As shown in Fig. 16, equilibrium dissociation constant (Kd) was estimated to be 1.6±0.2μM (n=3). This value was similar to that reported in brain synaptosomes (J. Neurochem. 60, 1191-1201, 1993, Life Sci. 35, 2335-2343, 1984, Brain Res. 348, 321-330, 1985). Further, displacement of specific [^{3}H] HC3 binding by HC3, choline (Cho) and acetylcholine (Ach) was examined. Acetylcholine was measured in the presence of 1 μM physostigmine. The result is shown in Fig. 17. Fig. 17 indicates that specific [^{3}H] HC3 binding was displaced when the concentration of choline was at least about 10 times lower than that of acetylcholine. These results show that CHT1 is a HC3 binding site as well as a high-affinity choline transporter.

[0021]

The cDNA of human high-affinity choline transporter of the present invention, being represented by Seq. ID No.5, can be prepared, for example, as follows: data base search was conducted with the amino acid sequence of nematode (C. elegans) CHO-1 to find a sequence of specific human genome DNA fragment having significant homology (R-107P12, a clone of human genomic survey sequence; GenBank accession number: AQ316435); a gene-specific primers for PCR were designed based on a base

sequence of said DNA fragment; 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE were conducted using Marathon-Ready™ cDNA (Clontech) of human whole brain, together with an attached adapter primer; the obtained PCR product was cloned into a cloning vector for PCR, and a base sequence of inserted DNA was determined. In addition, an amino acid sequence expected from this DNA sequence is represented by Seq. ID No. 6. A protein having human high-affinity choline transporter activity represented by said Seq. ID No. 6 can be constructed by a usual method on the basis of DNA sequence information shown in Seq. ID No. 5.

[0022]

As examples of a protein having high-affinity choline transporter activity of the present invention, in addition to the ones represented by Seq. ID Nos. 2, 4, and 6, which are specifically disclosed above, a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino acid sequences represented by Seq. ID Nos. 2, 4, and 6, and having high-affinity choline transporter activity is also included. These proteins can be prepared by known methods. Further, examples of a gene or DNA encoding a protein having high-affinity choline transporter activity of the present invention include, in addition to the ones represented by Seq. ID Nos. 1, 3, and 5, which are specifically disclosed above, a gene or DNA which encodes a protein comprising an amino acid sequence wherein one or a few amino acids are deficient, substituted or added in amino acid sequences represented by Seq. ID Nos. 2, 4, and 6, and having high-affinity choline transporter activity, and DNA which encodes a protein hybridizing with said gene or DNA under a stringent condition and having high-affinity choline transporter activity. These

genes and DNAs can be prepared by known methods.

[0023]

Cholinergic neurons play an extremely important role in learning and memory. The damage of these neurons correlates to severity of dementia. The rate-limiting step in acetylcholine synthesis is presumed to be the uptake of choline, and its activity is controlled by neural activity or various kinds of stimuli. In the brains of patients who suffer Alzheimer's disease, the hyperfunction of high-affinity choline uptake and of HC3 binding activity are observed (Trends Neurosci. 15, 117-122, 1992, Ann. NY Acad. Sci. 777, 197-204, 1996, J. Neurochem. 69, 2441-2451, 1997). Cloning of said gene or DNA encoding a protein having high-affinity choline transporter activity and said protein having high-affinity choline transporter activity is important for elucidating the molecular mechanism of the high-affinity choline transporter and for developing new therapies for Alzheimer's disease.

[0024]

An antibody that specifically combines with a protein having high-affinity choline transporter activity of the present invention can be constructed by a usual method with the above-mentioned protein having high-affinity choline transporter activity as an antigen, and a monoclonal antibody is preferable because of its specificity. Said monoclonal antibody is useful, for instance, for elucidating molecular mechanism of regulation of a high-affinity choline transporter.

[0025]

By using the gene or DNA that encodes a protein having high-affinity choline transporter activity, or a protein having high-affinity choline transporter activity, it becomes possible

to screen a pharmaceutical material useful for the treatment of Alzheimer's disease, in other words, a material that promotes or suppresses the activity or the expression of a high-affinity choline transporter. The screening method of the present invention include a method wherein high-affinity choline transporter activity of a protein having high-affinity choline transporter activity of the present invention is evaluated in the presence of a subject material, a method wherein a cell which can express a protein having high-affinity choline transporter activity of the present invention is cultivated in vitro in the presence of a subject material, and the activity and/or the expression amount of a protein having high-affinity choline transporter activity in the cell is evaluated, and a method wherein a subject material is administered to a non-human animal and then the activity and/or the expression amount of a protein having high-affinity choline transporter activity in a cell which can express a protein having high-affinity choline transporter activity of the present invention is evaluated.

[0026]

In addition, cells being useful for gene therapy of Alzheimer's disease and the like can be prepared by using a whole or a part of a gene or DNA that encodes a protein having high-affinity choline transporter activity of the present invention. As an example of a method for preparing these cells of the present invention, a method wherein a whole or a part of said gene or DNA of the present invention is introduced into a cell being deficient in the function of a high-affinity choline transporter gene on its chromosome by transfection or the like to obtain a cell having high-affinity choline transporter activity is exemplified. As the cell having high-affinity

choline transporter activity, in particular, it is preferable to use a cell wherein said gene or DNA is integrated into a chromosome and high-affinity choline transporter activity is exhibited stably.

[0027]

In the present invention, said non-human animal whose function of a high-affinity transporter gene is deficient on its chromosome means a non-human animal wherein a part or a whole of a high-affinity choline transporter gene on chromosome is inactivated by gene mutation such as disruption, deficiency, substitution, etc. and function of expressing a high-affinity transporter is lost. In addition, a non-human animal that abnormally expresses function of a high-affinity transporter gene on its chromosome means a non-human animal that produces larger amount of a protein having high-affinity transporter activity than a wild-type non-human animal does. Though specific examples of a non-human animal of the present invention include rodents, such as mice, rats and the like, a non-human animal of the present invention is not limited to these animals.

[0028]

In the present invention, wild-type non-human animal means an animal of the same species of said non-human animals whose function of a high-affinity transporter gene is deficient on its chromosome, and a littermate is preferable. Homozygous mice generated according to Mendelian ratio include a deficient type for a high-affinity choline transporter and their littermate wild-type, and it is possible to carry out precise comparative experiments in individual level by using the deficient types and the littermate wild-types of these homozygous mice at the same time. The generating method of the non-human animals being

deficient in the function of a high-affinity transporter gene on their chromosome will be explained below, with an example of knockout mice being deficient in a high-affinity transporter.

[0029]

For example, a mouse being deficient in the function of a high-affinity transporter gene on its chromosome, in other words, a knockout mouse of a high-affinity transporter can be constructed as follows. A high-affinity transporter gene is screened by using a gene fragment obtained from mouse gene library by a method like PCR. The screened high-affinity transporter gene is subcloned with a viral vector or the like, and specified by DNA sequencing. A target vector is constructed by substituting a whole or a part of a high-affinity transporter gene of this clone with pMC1 neo gene cassette or the like, and by introducing a diphteria toxin A fragment (DT-A) gene, a herpes simplex virus thymidine kinase (HSV-tk) gene or other such genes into 3'-terminal side.

[0030]

This constructed target vector is linearized and introduced into ES cells by electroporation or the like to induce homologous recombination. The ES cells wherein homologous recombination is induced by an antibiotic such as G418, ganciclovir (GANC) or the like are selected from the homologous recombinants. It is preferable to confirm whether the selected ES cells are the recombinants of the object by Southern blot or the like. A chimeric mouse is constructed by microinjecting a clone of the confirmed ES cells into a blastocyst of a mouse and then transplanting the blastocyst into a recipient mouse. A heterozygous mouse can be obtained by interclossing the chimeric mouse with a wild-type mouse, and a knockout mouse of

a high-affinity transporter of the present invention can be constructed by interclossing the heterozygous mice. It is possible to confirm whether a knockout mouse of a high-affinity transporter is constructed, for example, by isolating RNA from the mouse obtained by said method and examining it by Northern blot analysis or the like, or by examining the expression of the mouse by Western blot analysis or the like.

[0031]

Further, a promoter or a suppressor of high-affinity choline transporter activity, or of high-affinity choline transporter expression can be screened by administering a subject material to said knockout mouse of a high-affinity transporter and then evaluating the high-affinity transporter activity or the like in the knockout mouse.

[0032]

Experimental methods or the like of the above-mentioned various experiments will now be explained in more detail below.

(Cloning of transporter cDNA)

The candidate cDNA of nematode choline transporter was isolated from poly (A)+RNA of nematode mixture from various stages in the development by reverse transcription PCR and 3' RACE. Marathon™ cDNA Amplification Kit (Clontech) was used according to its protocol. A primer for sense direction of PCR was designed at a provisional translation initiating point of a predicted gene based on a DNA base sequence obtained from C. elegans genomic project. The amplified PCR product was subcloned into Nco I (smoothing) site and Not I site of a modified pSPUTK vector (Stratagene), and the base sequence of inserted DNA was determined. CHT1 cDNA of rat was isolated from rat spinal cord cDNA library by using GeneTrapper cDNA Positive Selection

System (GIBCO Bio-Rad Laboratory: GIBCO BRL) according to its protocol. The primer used was designed from the base sequence of a cDNA fragment obtained by degenerated PCR. The obtained cDNA clones were analyzed. Among them, positive clones were selected and subcloned into pSPUTK vector and pcDNA3.1+ vector (Invitrogen Corporation).

[0033]

(Expression in oocytes of Xenopus)

In the presence of cap analog, cRNA was synthesized in vitro with SP6 or T7 RNA polymerase. 20 to 30 ng capped RNA was microinjected into oocytes (stage V to VI) of Xenopus. The uptake was measured in basically same manner as described previously (Nature 360, 467-471, 1992). Two or three days after the injection of RNA, choline uptake was conducted for 30 to 60 min. with oocytes (6 to 8) in 0.75 ml standard solution (0.01 to 1 μM [^{3}H]-choline, 100 mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 5mM Tris: pH 7.4). The oocytes completing uptake were solubilized with 10% SDS, and the amount of [^{3}H] was measured by a liquid scintillation counter.

[0034]

(GFC expression construct)

The transcriptional fusion construct of cho-1::gfp was constructed by PCR in same manner as described previously (Gene 212, 127-135, 1998). A gene that encodes a green fluorescent protein (GFP) located on downstream of a nuclear localization signal sequence (NLS) was inserted into a position 3 residues downstream of cho-1 translation initiating point so that the reading frame was fitted. NLS and gfp gene were amplified from pPD104.53 vector. In order to prepare 5.1 kb upstream region of cho-1 translation initiating point, a PCR primer being

designed to encompass the first 3 amino acid residues of cho-1 was used. By the same method as previously described (EMBO J. 10, 3959-3970, 1991), rol-6 (su1006) marker and generated DNA were injected into gonads of a nematode simultaneously.

【0035】

(Northern blot analysis)

6 μg poly(A)+RNA prepared from various tissues of rats was separated by formaldehyde-agarose electrophoresis, and transferred to a nylon membrane, then hybridized with CHT1 cDNA fragment being labeled with [^{32}P] by random prime method in hybridization solution (solution containing the final concentration of 50% formamido, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 μg/ml salmon sperm DNA) at 42° C for 16 hours. The nylon membrane was washed under final condition (0.1 × SSPE, 0.1% SDS: 65° C), and then autoradiography was conducted for 7 days together with an enhancing screen.

【0036】

(In situ hybridization)

The transcript of an antisense labeled with digoxigenin was synthesized in vitro. Alkaline hydrolysis was repeated for the transcripts until their mean length was prepared to be 200 to 400 b. Cryostat sections of fresh frozen tissue (10 to 20 μm) were used. Hybridization was conducted with labeled cRNA probe (about 1 μg/ml) dissolved in 1 × Denhardt's solution [solution containing the final concentration of 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.3 M NaCl, 50% formamido, 10% dextran sulphate, 1 mg/ml E. coli tRNA] at 45° C for 20 hours. Then the sections were washed twice in 2 × SSC/50% formamido and once in 1 × SSC/50% formamido, at 45° C respectively. The hybridized probe was visualized by using anti-digoxigenin Fab fragment

(Boehringer-Mannheim) and NBT/BCIP substrate. The sections were brought into reaction in substrate solution for 24 to 48 hours.

[0037]

(Binding assay)

[^{3}H] hemicholinium-3 (HC3; 128Ci/mmol) was obtained from NEN Life Science Products. Either pcDNA3.1-CHT1 or pcDNA3.1 was transiently expressed in COS7 cells respectively. TransFast Reagent (Promega) was introduced and used according to the protocol. Membranes were prepared by following steps: homogenizing cells in 0.32 M sucrose; centrifuging the cells for 1 hour at 200,000g; and suspending the precipitate. Binding assay was conducted in basically same manner as described previously. Specific binding amount was calculated by subtracting non-specific binding amount determined in the presence of 10 μM HC3 from the whole binding amount. The Kd value was figured out by analyzing specific [^{3}H] HC3 binding amount from data of saturation binding assay with nonlinear approximation.

[0038]

[Effect of the Invention]

The present invention makes it possible to provide a protein having high-affinity choline transporter activity, which is physiologically important, and gene DNA encoding said protein. In addition, by using the said protein and gene DNA, it becomes possible to screen materials being useful for prevention or treatment of Alzheimer's disease, and to prepare cells being useful for gene therapy.

[0039]

[Sequence Listing]

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            20                  25                  30

Gly Asn Ala Glu Glu Arg Ser Glu Ala Ile Ile Val Gly Gly Arg Asp
        35                  40                  45

Ile Gly Leu Leu Val Gly Gly Phe Thr Met Thr Ala Thr Trp Val Gly
    50                  55                  60

Gly Gly Tyr Ile Asn Gly Thr Ala Glu Ala Val Tyr Gly Pro Gly Cys
65                  70                  75                  80

Gly Leu Ala Trp Ala Gln Ala Pro Ile Gly Tyr Ser Leu Ser Leu Ile
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Leu Gly Gly Leu Phe Phe Ala Lys Pro Met Arg Ser Lys Gly Tyr Val
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Thr Met Leu Asp Pro Phe Gln Gln Ile Tyr Gly Lys Arg Met Gly Gly
        115                 120                 125

Leu Leu Phe Ile Pro Ala Leu Met Gly Glu Met Phe Trp Ala Ala Ala
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Val Asn Ile Ser Val Ile Val Ser Ala Leu Ile Ala Ile Leu Tyr Thr
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Leu Val Gly Gly Leu Tyr Ser Val Ala Tyr Thr Asp Val Val Gln Leu
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Phe Cys Ile Phe Ile Gly Leu Trp Ile Ser Val Pro Phe Ala Leu Ser
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His Pro Val Val Thr Asp Ile Gly Phe Thr Ala Val His Ala Lys Tyr
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Gln Ser Pro Trp Leu Gly Thr Ile Glu Ser Val Glu Val Tyr Thr Trp
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Leu Asp Asn Phe Leu Leu Leu Met Leu Gly Gly Ile Pro Trp Gln Ala
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Tyr Phe Gln Arg Val Leu Ser Ser Ser Ser Ala Thr Tyr Ala Gln Val
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Ile Cys Ile Gly Ala Ile Gly Ala Ser Thr Asp Trp Asn Gln Thr Ala

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Tyr Gly Phe Pro Asp Pro Lys Thr Lys Glu Glu Ala Asp Met Ile Leu
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Gly Ser Ala Glu Glu Arg Ser Glu Ala Ile Ile Val Gly Gly Arg Asp
        35                  40                  45

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Ile Gly Leu Leu Val Gly Gly Phe Thr Met Thr Ala Thr Trp Val Gly
    50                  55                  60

gga ggg tat atc aat ggc aca gct gaa gca gtt tat gta cca ggt tat   240
Gly Gly Tyr Ile Asn Gly Thr Ala Glu Ala Val Tyr Val Pro Gly Tyr
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Leu Val Gly Gly Leu Tyr Ser Val Ala Tyr Thr Asp Val Val Gln Leu
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His Pro Ala Val Ala Asp Ile Gly Phe Thr Ala Val His Ala Lys Tyr
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Gln Lys Pro Trp Leu Gly Thr Val Asp Ser Ser Glu Val Tyr Ser Trp
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Leu Asp Ser Phe Leu Leu Leu Met Leu Gly Gly Ile Pro Trp Gln Ala
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Tyr Phe Gln Arg Val Leu Ser Ser Ser Ser Ala Thr Tyr Ala Gln Val
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Asp Asn Leu Gln

580

【Brief Explanation of the Drawings】

【Fig. 1】

This is a view showing the result of [^{3}H] choline uptake of oocytes from Xenopus of the present invention being injected with C. elegans cho-1 (C48D1.3 cRNA) or water.

【Fig. 2】

This is a view showing the result of the effect of Na^+ on choline uptake of oocytes from Xenopus of the present invention being injected with C. elegans cho-1 (C48D1.3 cRNA) or water.

【Fig. 3】

This is a view showing the result of the HC3-induced inhibition of choline uptake of oocytes from Xenopus of the present invention being injected with C. elegans cho-1 (C48D1.3 cRNA) or water.

【Fig. 4】

This is a view showing amino acid sequences of rat CHT1 and C. elegans CHO-1 of the present invention respectively.

【Fig. 5】

This is a view showing the distribution of neurons expressing cho-1::gfp of the present invention in the nervous system of C. elegans.

【Fig. 6】

This is a view showing the phylogenetic tree of Na^+-dependent glucose transporter family.

【Fig. 7】

This is a view showing an expected topology of rat CHT1

of the present invention.

[Fig. 8]

This is a view showing the result of Northern blot analysis of CHT1 mRNA transcript in rat tissue of the present invention.

[Fig. 9]

This is a view showing the result of *in situ* hybridization analysis of CHT1 transcript in a rat brain of the present invention.

[Fig. 10]

This is a view showing the result of *in situ* hybridization analysis of CHT1 transcript in a spinal cord of the present invention.

[Fig. 11]

This is a view showing the result of [^{3}H] choline uptake of oocytes from Xenopus of the present invention being injected CHT1 cRNA of the present invention or water.

[Fig. 12]

This is a view showing the effect of choline concentration on choline uptake in CHT1 of the present invention.

[Fig. 13]

This is a view showing the result of HC3-induced inhibition of choline uptake of CHT1 of the present invention.

[Fig. 14]

This is a view showing the result of Na^+- and Cl^--dependent choline uptake of CHT1 of the present invention.

[Fig. 15]

This is a view showing the result of [^{3}H] HC3 binding to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention or vector pcDNA 3.1 separately.

[Fig. 16]

This is a view showing the result of saturation analysis of specific [^{3}H] HC3 binding to the membrane prepared from COS7 cells being introduced with CHT1 cDNA of the present invention or vector pcDNA 3.1 separately.

[Fig. 17]

This is a view showing the result of displacement of specific [^{3}H] HC3 binding by HC3 of the present invention, choline (Cho), acetylcholine (ACh).

【Name of Document】 ABSTRACT

【Abstract】

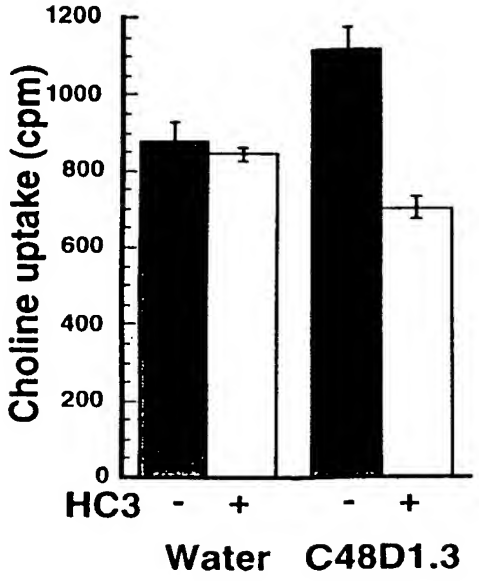
【The Object】 The object of the present invention is to provide a protein having high-affinity choline transporter activity which is important physiologically, a gene encoding the protein, and a method of screening a material promoting the high-affinity choline transporter activity with the use of the same, and the like.

【Solving Means】 By examining high-affinity choline uptake activity of Na^+-dependent transporter cDNA deduced from the genomic sequence of a nematode C.elegans in a Xenopus oocyte expression system, the cDNA (cho-1) of nematode high-affinity choline transporter is identified. Then the cDNA (CHT1) of rat high-affinity choline transporter is cloned from rat spinal cord by using the homology of a base sequence to this cDNA as an index. Similarly, the cDNA of human high-affinity choline transporter is cloned from human genome.

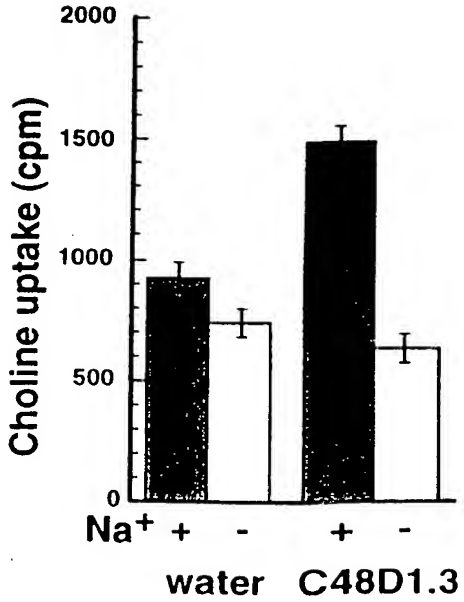
【Selected Drawing】 Fig. 4

[Name of Document] Drawing

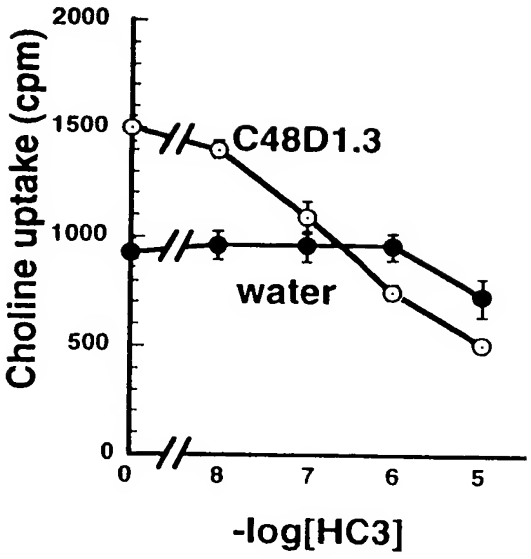
[Fig. 1]



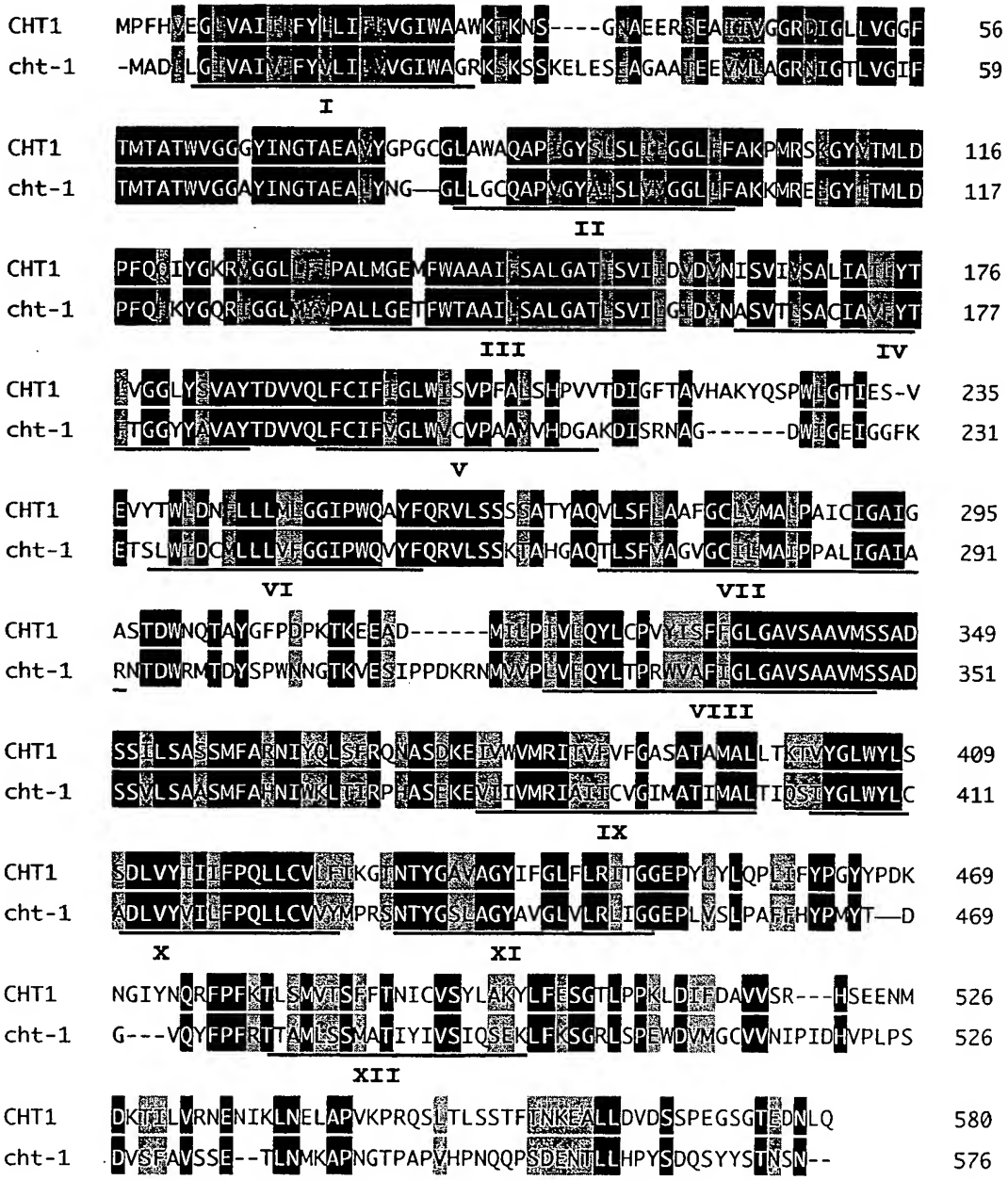
[Fig. 2]



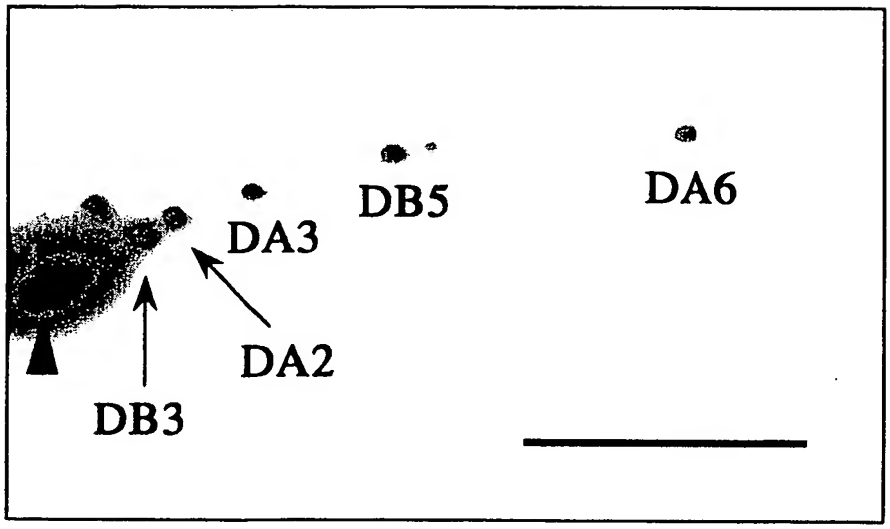
[Fig. 3]



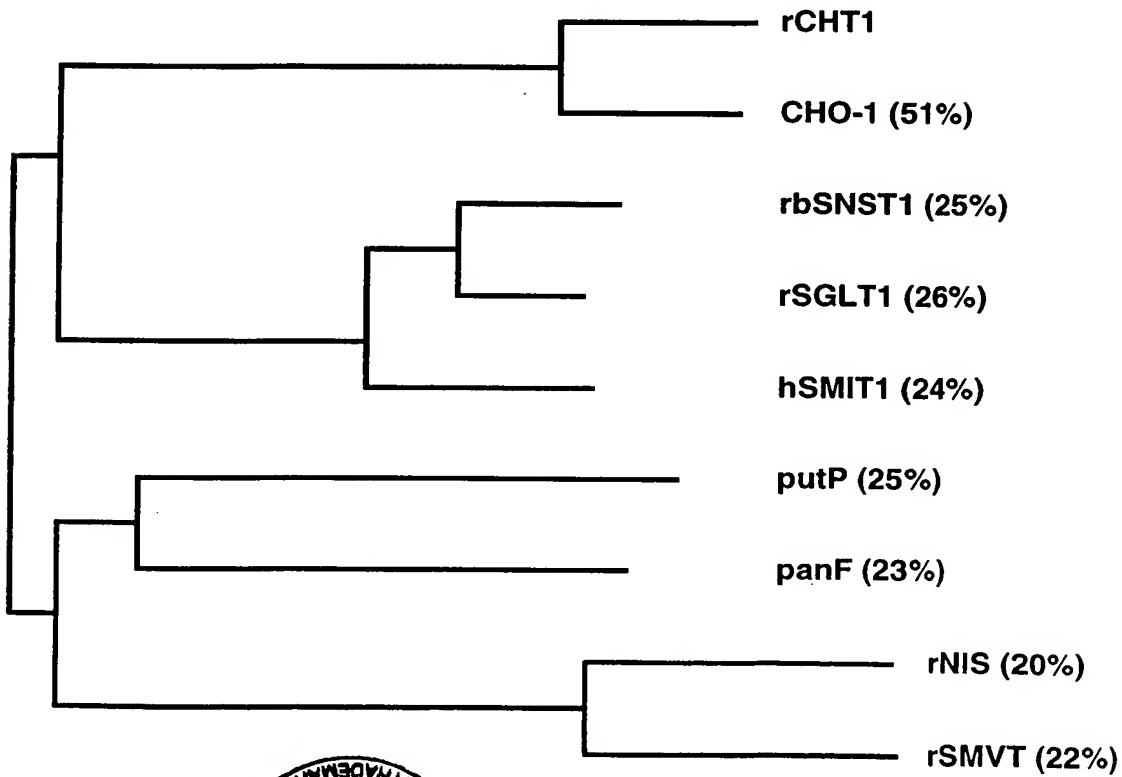
【Fig. 4】



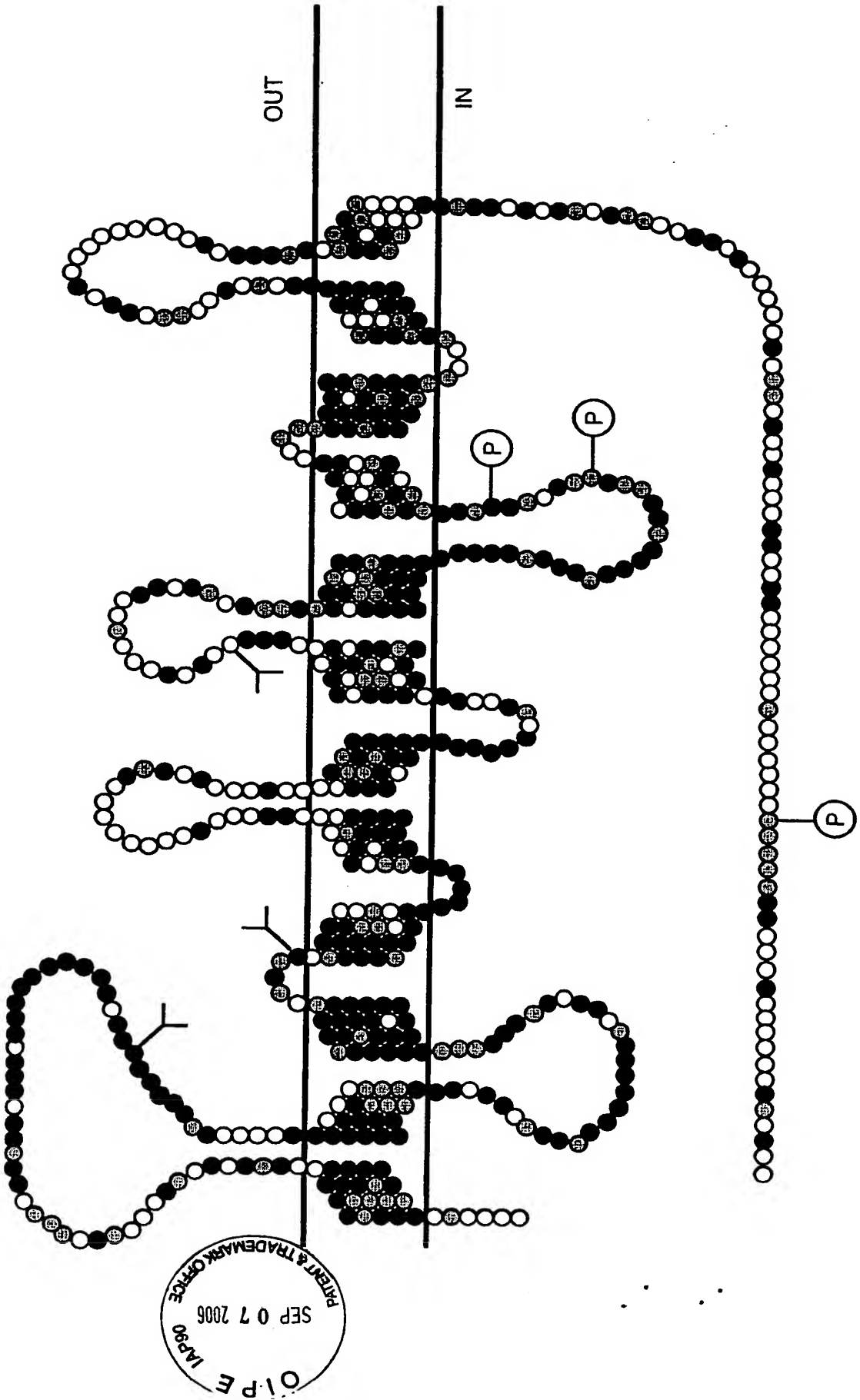
[Fig. 5]



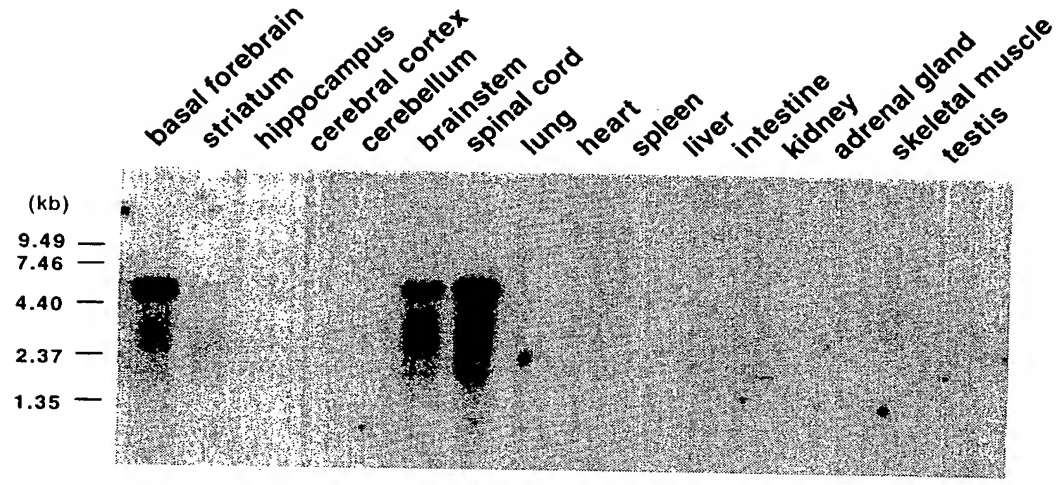
[Fig. 6]



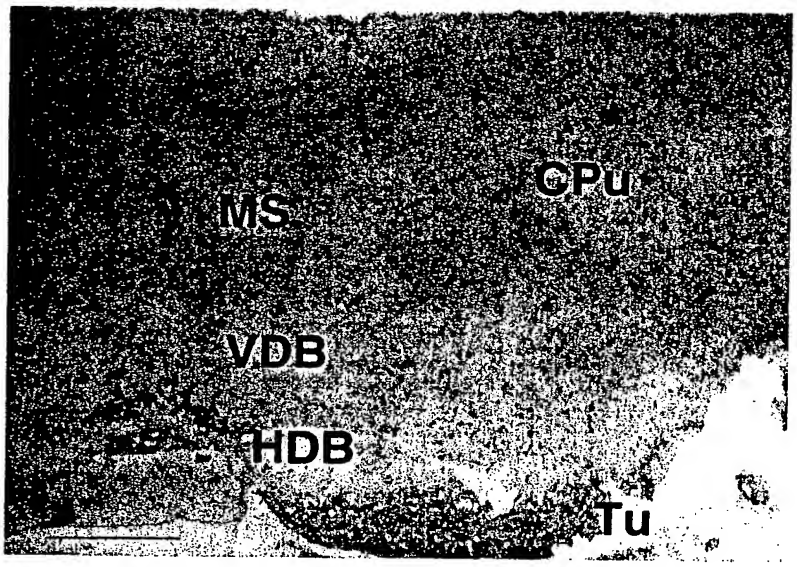
【Fig. 7】



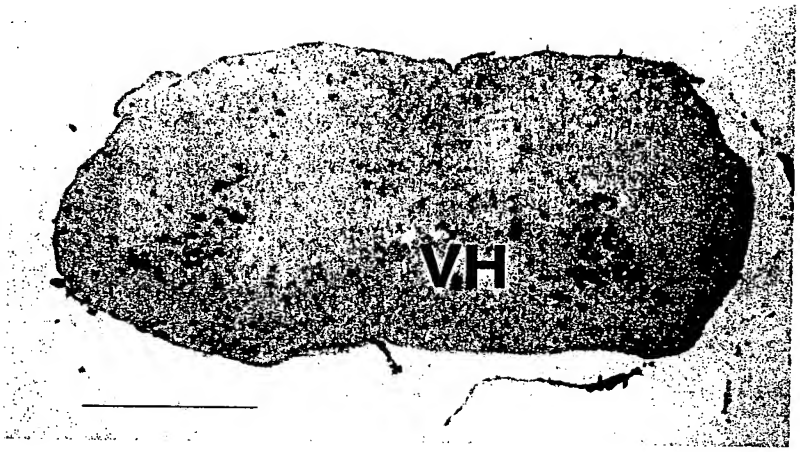
【Fig. 8】



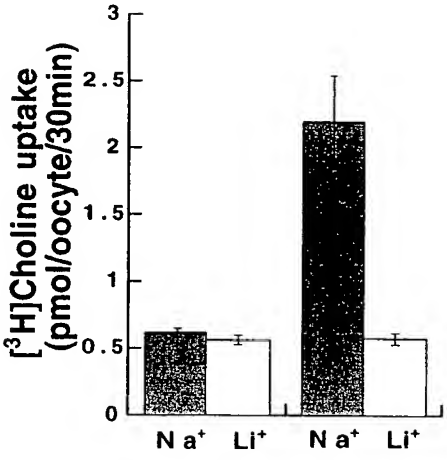
【Fig. 9】



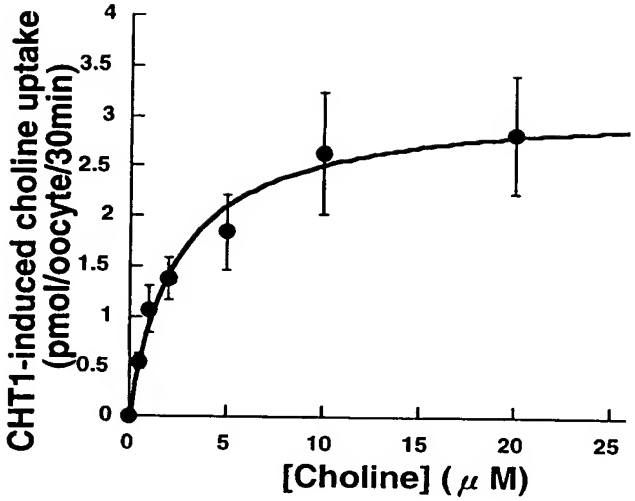
【Fig. 10】



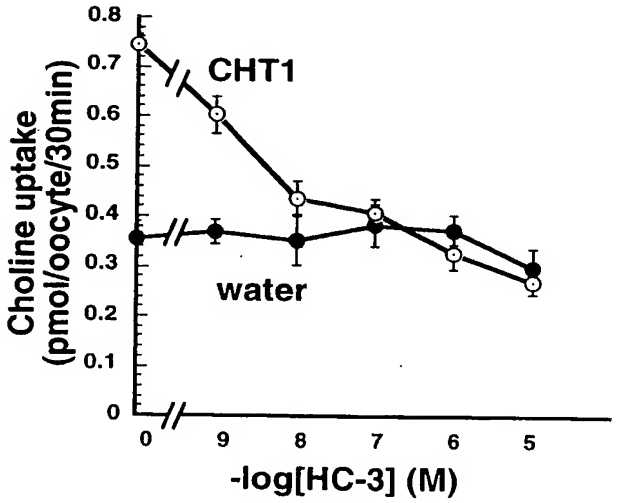
【Fig. 11】



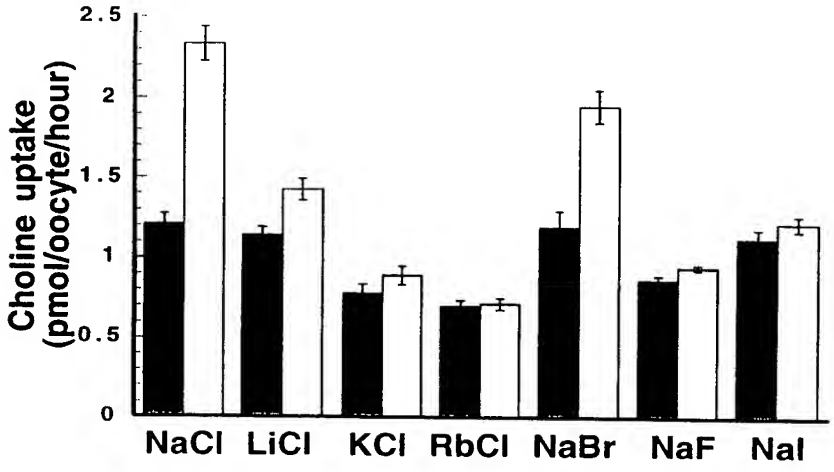
[Fig. 12]



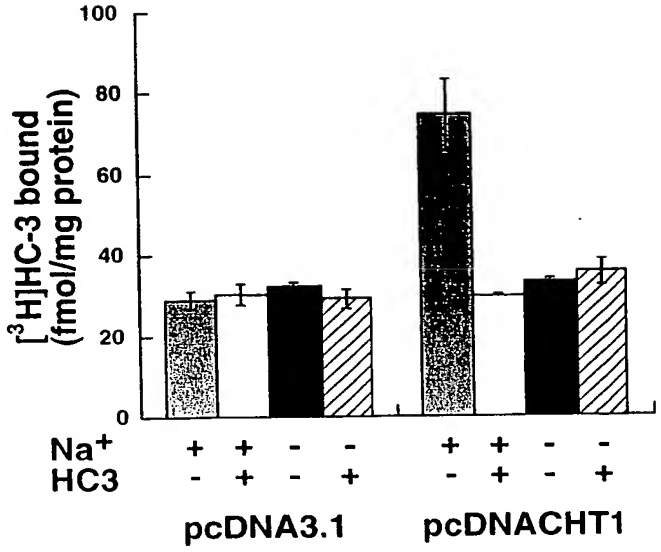
[Fig. 13]



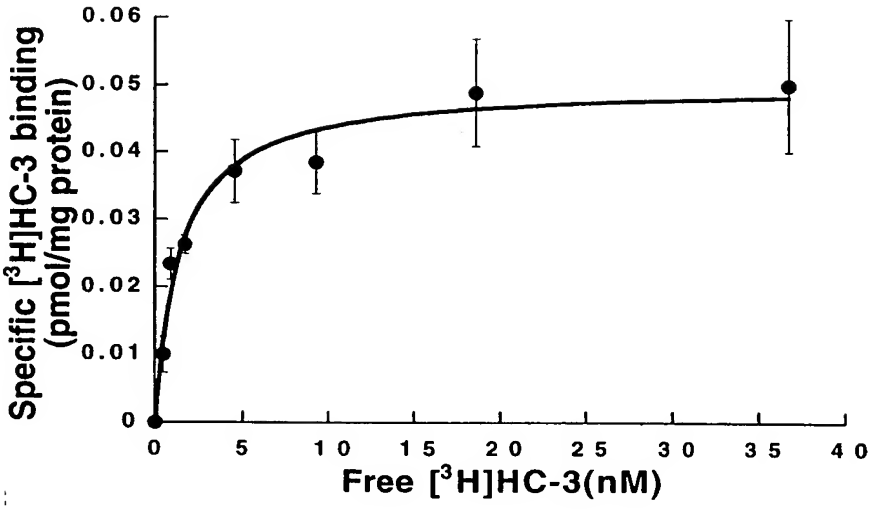
【Fig. 14】



【Fig. 15】



[Fig. 16]



[Fig. 17]